

## ORIGINAL PAPER

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## Genome analysis of the moss *Physcomitrella patens* (Hedw.) B.S.G.<sup>1</sup>

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**Abstract** A wild-type (WT) strain of the moss *Physcomitrella patens* (Hedw.) B.S.G., two mutants derived from it (PC22 and P24), and a somatic hybrid, PC22(+)P24, were analysed. Staining of metaphases revealed  $54 \pm 2$  chromosomes in the somatic hybrid and 27 chromosomes in the wild type and the two mutants. Using flow cytometry (FCM), DNA contents were calculated to be 0.6 pg (WT, PC22), 1.2 pg (P24), and 1.6 pg (PC22(+)P24) per nucleus, respectively. Southern hybridization provided evidence for at least one family of highly repetitive DNA and, furthermore, revealed different amounts of repetitive DNA in the four genotypes. However, these sequences cannot account for the 100% increase in the nuclear DNA amount in mutant P24, relative to wild type. In FCM analyses every moss genotype generated just one single peak of fluorescence, indicating an arrest in the cell cycle during the daytime. Thermal denaturation of wild-type DNA revealed a G+C content of 34.6% for total DNA and 38.6% for plastid DNA. A cDNA library of  $1.2 \times 10^6$  independent clones was established, from which sequences homologous to *cab* and *rbcS*, respectively, were isolated. These genes show significant homologies to those of higher plants, and, likewise, comprise multigene families. No restriction fragment length polymorphisms could be detected between the four moss genotypes using these cDNA probes.

**Key words** Archegoniate · Differentiation mutant  
Plant nuclear DNA · Repetitive DNA  
Somatic hybrid

### Introduction

In the first few decades of this century, bryophytes were at the forefront of genetical research: Allen (1917) was the first to describe sex chromosomes in plants, Heitz (1928) demonstrated the continuity of chromosomes during the mitotic cell cycle, and Knapp (1935, 1936, 1937) employed X-ray mutagenesis for genetic studies. Although the beginning of plant molecular genetics was marked by UV mutagenesis of *Sphaerocarpos*, demonstrating DNA as the molecular basis of inheritance (Knapp et al. 1939), bryophytes later became of marginal interest for molecular biologists. The outstanding exception is the liverwort *Marchantia polymorpha*, which was the subject of intensive mutagenesis and cross-breeding experiments (Burgeff 1943), and is now well known, since the complete nucleotide sequences of its plastid and mitochondrial DNAs have been determined (Ohyama et al. 1986; Oda et al. 1992).

Non-Mendelian inheritance was first postulated by von Wettstein (1928) based on analysis of *Funaria hygrometrica* and related mosses. Likewise, von Wettstein (1932) recognised the great potential of haploid moss protonemata for genetically dissecting differentiation processes. Such analyses started with mutant induction of *Physcomitrium piriforme*, *F. hygrometrica*, and *Physcomitrella patens* (Barthelmess 1940; Oehlkers and Bopp 1957; Engel 1968) and culminated in synopses on the physiology and genetics of cellular differentiation in *Funaria* and *Physcomitrella* (Bopp 1981, 1990; Cove 1992; Cove and Knight 1993). These studies led to the suggestion that genetic dissection of *Physcomitrella* could make significant contributions to the understanding of the evolution of plant developmental processes (Goldberg 1988; Chasan 1992). However, knowledge of the genetics of mosses is still fragmentary, thus impeding broader exploitation of their potential.

We have established a series of protocols for the purification of all three organellar DNAs from *Physcomitrella* (Marienfeld et al. 1989), and thus were the first to analyse the mitochondrial genes of an

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archegoniate (Marienfeld et al. 1991, 1992) and the plastid genes of a moss (Kasten et al. 1991, 1992). Nevertheless, information on nuclear DNA of mosses is still scarce, although an important breakthrough was achieved with the stable transformation of *Physcomitrella* (Schaefer et al. 1991). However, there is virtually no data on the complexity of its genome and even the chromosome number is uncertain, figures varying from  $n=14$  (Engel 1968) to  $n=27$  (Bryan 1957). In the present article we report on our analyses of the *P. patens* genome.

## Materials and methods

### Plant material

The *P. patens* (Hedw.) B.S.G. wild-type used in our studies was propagated vegetatively and by self-pollination. It is a subculture of strain 16/14 which was collected by H.L.K. Whitehouse in Gransden Wood, Huntingdonshire, UK and was propagated by Engel (1968) from a single spore. For mutant induction, spores were incubated for 24 h in water, treated with X-rays at 1 kR/min for 40 min and transferred to unsupplemented mineral medium. Anomalous growing plants were isolated (Fiedrich and Abel, unpublished). Two of these are the cytokinin-sensitive differentiation mutants PC22 and P24; PC22 is impeded in bud formation and chloroplast division (Abel et al. 1989), P24 is impeded in bud formation (Ye et al. 1989). Hansen et al. (1988) generated a somatic hybrid PC22(+)P24 by one-to-one protoplast fusion.

### Culture conditions

Plants were grown under sterile conditions in agitated liquid medium (250 mg  $\text{KH}_2\text{PO}_4$ , 250 mg  $\text{KCl}$ , 250 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1000 mg  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 12.5 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , pH 5.8) in a growth chamber at  $25 \pm 1^\circ\text{C}$  under a light-dark regime of 16:8 h, with illumination starting at 6.00 a.m. Small-scale cultures were grown in 500 ml Erlenmeyer flasks containing 200 ml of culture medium and covered with silicone sponges (Belco, Vineland, N.J., USA). These flasks were shaken on a GFL shaker (Hannover, Germany; type 3015) set at 100 rpm and were illuminated from above by two fluorescent tubes (Philips TL 65W/25) providing  $55 \mu\text{E}/\text{m}^2$  per s outside the flasks. For large-scale cultures, a 14-day-old 200-ml culture was disintegrated, using an Ultra-Turrax homogenizer (IKA, Staufen, Germany), into fragments containing 5–10 cells. This suspension was used to inoculate 5 l medium in a 6 l culture vessel closed with a wad of tissue paper and ventilated with sterile compressed air. These cultures were illuminated from above by two fluorescent tubes (Philips TL 65W/25) providing  $32 \mu\text{E}/\text{m}^2$  per s outside the vessels at medium height.

### Chromosome staining

Protonemata were grown for 3 weeks in small-scale cultures as described above, changing the medium twice a week and disintegrating the plants once a week with an Ultra-Turrax. Four days after the last disintegration, 10 mg *p*-dichlorobenzene and a droplet of  $\alpha$ -bromonaphthalene were added to 25 ml medium. After 3 h, mosses were collected in a sieve, rinsed several times with water and transferred for 2 h into a solution of 2% Driselase (Sigma) in 0.5 M mannitol. Protoplasts were isolated according to Rother et al. (1994), and were treated and stained with Giemsa according to Wang et al. (1992). Meiotic chromosomes were analysed as follows in the fertile wild-type strain: formation of sex organs was induced by incubation at  $15^\circ\text{C}$ , and plants were rinsed with sterile water to assure sporogone formation. Capsules

were fixed in fresh 3:1 methyl alcohol acetic acid for 3 h at room temperature and stored at  $-20^\circ\text{C}$ . Chromosomes were stained with the iron-acetocarmine method according to Belling (1926), cells were fixed in Hoyer's medium (Bowen 1963) and spore mother cells were isolated by squeezing.

### Thermal denaturation

Samples containing 50  $\mu\text{g}/\text{ml}$  CsCl-purified total DNA or plastid DNA, in SSC (0.15 M NaCl, 15 mM sodium citrate) were heated from 60 to  $99^\circ\text{C}$  at the rate of  $1^\circ\text{C}/\text{min}$  in an Gilford Thermal Programmer (type 2527); absorption was measured at 260 nm every minute and was recorded by a plotter (Hewlett Packard, type 7225B). The G+C content was inferred from the respective melting temperature ( $T_m$ ; Marmur and Doty 1962).

### Flow cytometry

Flow cytometry (FCM) was carried out according to de Laat et al. (1987) and Ulrich and Ulrich (1991) in a Partec Cell Analyser CAII with 4',6-diamidino-2-phenylindole (DAPI) stained material.

### cDNA-cloning

RNA was isolated from 9-day-old wild-type protonemata according to Reski et al. (1991) and poly(A)<sup>+</sup> RNA was enriched on oligo(dT)-cellulose according to standard procedures (Sambrook et al. 1989). Five micrograms of polyadenylated RNA was used to construct a cDNA library (cDNA synthesis kit, Boehringer Mannheim, Germany) in  $\lambda\text{GT}10$  (Sambrook et al. 1989). About 1000 clones were plated, transferred onto nylon membranes and screened with radiolabelled probes for *cab* (from *Pisum sativum*; Cashmore 1986) and *rbcS* (from *Solanum tuberosum*; Wolter et al. 1988), respectively. Some positive clones were subcloned as *EcoRI* inserts into pBluescript in *Escherichia coli* XL-1 Blue and subjected to partial sequence analysis.

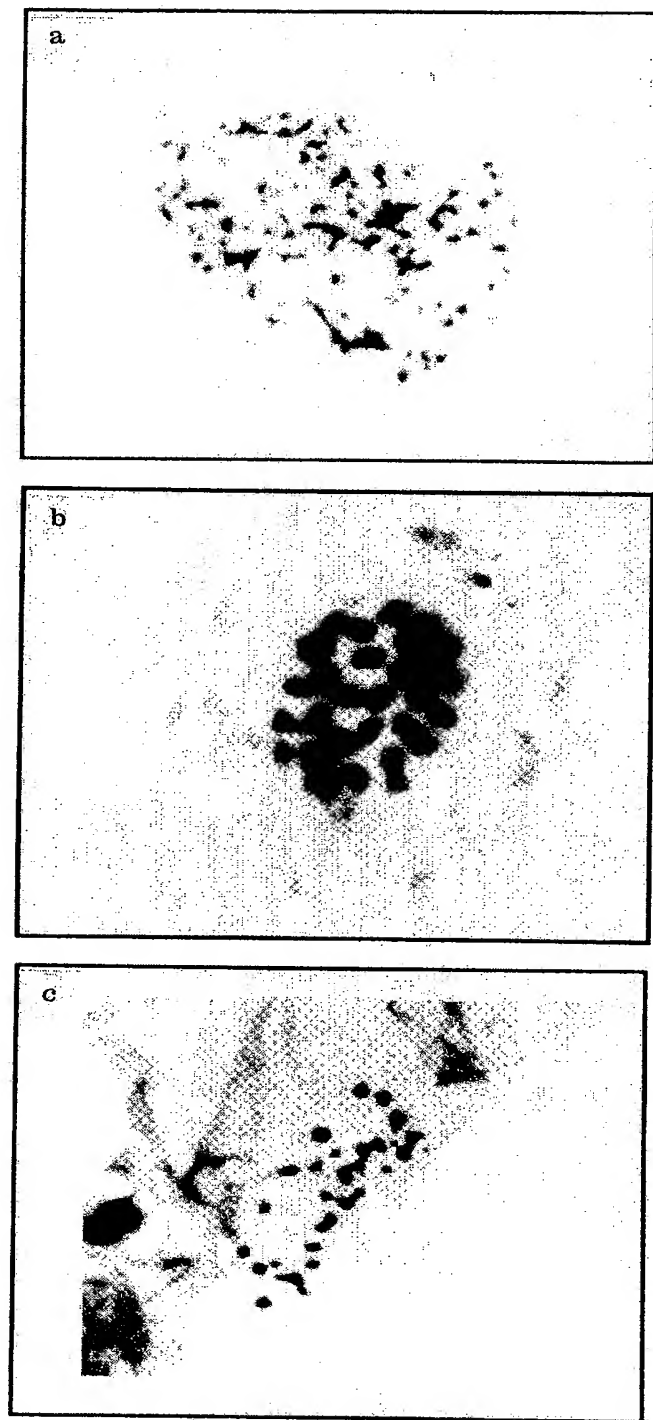
### DNA isolation and Southern analyses

These were carried out as previously described (Marienfeld et al. 1989; Reski et al. 1991) from plants grown in large-scale cultures as described above. For restriction fragment length polymorphism (RFLP) analyses, 3  $\mu\text{g}$  total DNA per lane was used. The homologous probes were labelled with the DIG (digoxigenin) random-primed labelling kit (Boehringer Mannheim, Germany).

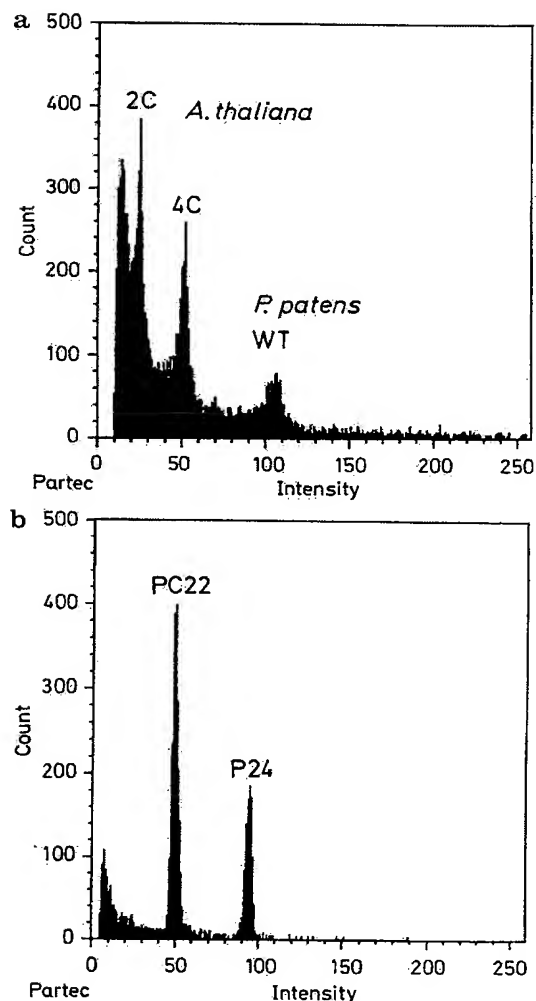
## Results

### Meiotic and mitotic chromosomes

The fusant PC22(+)P24 has larger cells, larger nuclei, and more actively dividing cells than the wild type or the two mutants. Analysis of ten metaphases by phase contrast microscopy revealed the chromosome number in PC22(+)P24 to be  $54 \pm 2$  (Fig. 1a). In the wild-type we counted at the most 27 meiotic bivalents, ranging in size from 1 to 2  $\mu\text{m}$  (Fig. 1b). Mitotic chromosomes of the moss were 0.5–1  $\mu\text{m}$  in size. Analysis of ten mitotic metaphases likewise yielded a value of  $n=27$  in the wild type (Fig. 1c), and in the two differentiation mutants PC22 and P24 (data not shown). For all genotypes, metaphases could be best accumulated between



**Fig. 1a-c** Chromosomes of a moss, *Physcomitrella patens*. **a** PC22(+ )P24: metaphase of mitosis. **b** Wild type: metaphase I of meiosis. **c** Wild type: metaphase of mitosis



**Fig. 2a, b** Flow cytometric analyses of 4',6-diamidino-2-phenylindole (DAPI)-stained isolated nuclei. The number of nuclei (x-axis) is plotted against the relative fluorescence (y-axis). **a** Comparison of *Arabidopsis thaliana* (2C; 4C) and *Physcomitrella patens* wild type. **b** Comparison of the two *Physcomitrella* mutants PC22 and P24

2.00 a.m. and 4.00 a.m. The chromosomes were too small to allow use of specific banding techniques.

#### DNA content and base composition

Nuclear DNA contents were calculated based on DAPI fluorescence using *Arabidopsis thaliana* and *Brassica napus* as references; these species contain 0.14 pg and 3.2 pg DNA per 2C nucleus, respectively (Bennet et al. 1982). In several independent experiments the moss genotypes were compared one to another and to the references. From this we inferred nuclear DNA contents of 0.6 pg (WT, PC22), 1.2 pg (P24) and 1.6 pg (PC22(+ )P24) (Fig. 2), corresponding to 600 Mbp (WT, PC22), 1,200 Mbp (P24) and 800 Mbp (PC22(+ )P24),

respectively, in  $G_1$  nuclei. However, all mosses exhibited only one peak in the FCM analyses, although we used fast-growing protonemata for these experiments and compared plants after different periods of subculture. The G + C content of total, and of plastid, DNA of the *Physcomitrella* wild type was calculated from thermal denaturation kinetics. We found the total DNA to have a G + C content of 34.6% ( $T_m$  value 83.5°C) and the plastid DNA to have a G + C content of 38.6% ( $T_m$  value 85.13°C).

### Repetitive DNA

Repetitive nuclear DNA was analysed by digesting 3  $\mu$ g of total DNA with *Rsa*I and hybridizing with two different DNAs. A 500 bp *Eco*RI fragment of the *Physcomitrella* 25S rDNA was cloned and sequenced (EMBL data bank accession number, EMBL X76633), and detected one single 1.1 kb band in each of the four moss genotypes. However, the intensities of the signals were different: whilst it was highest in WT and PC22, it was significantly reduced in P24 (Fig. 3). When total WT DNA, sonicated into fragments of approx. 500 bp, was used as a probe, at least one family of highly repetitive DNA was detected, revealing fragments of sizes from 100 bp to 4400 bp in every moss genotype. The amounts of this highly repetitive DNA appeared also to be variable, being highest in mutant P24 and lowest in the somatic hybrid (Fig. 4).

### cDNAs for *cab* and *rbcS*

Five micrograms of poly(A)<sup>+</sup>-enriched RNA were used to construct a cDNA library of approx.  $1.2 \times 10^6$  independent clones with insert sizes ranging from 350 bp to 7500 bp. Using *cab* from *P. sativum* as a probe in plaquelift hybridizations, 77 out of 1000 plaques hybridized. One of these contained a 750-bp insert with 97% amino acid homology to exon 2 of a genomic *Physcomitrella cab* sequence (EMBL M23532; Long

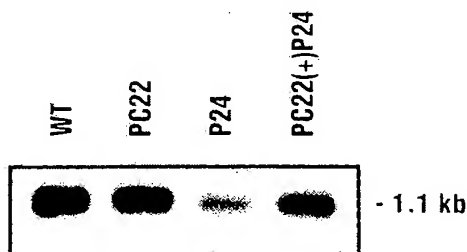


Fig. 3 Southern analyses of *Rsa*I-digested total DNA of four *Physcomitrella* genotypes hybridized to a 25S rDNA-specific homologous probe. In 3  $\mu$ g of total DNA the probe detects one signal of 1.1 kb, which exhibits different intensities in the wild type (WT), the two X-ray-derived mutants PC22 and P24 and their somatic hybrid PC22(+)/P24

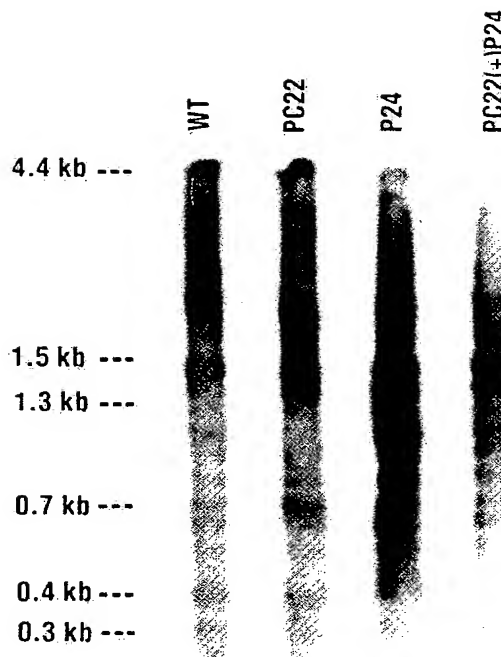


Fig. 4 Southern analyses of *Rsa*I-digested total DNA of four *Physcomitrella* genotypes hybridized to total wild-type DNA, which was sonicated into approx. 500-bp fragments. In 3  $\mu$ g of total DNA at least one family of repetitive DNA can be detected occurring in different amounts in the wild type (WT), the two X-ray-derived mutants PC22 and P24 and their somatic hybrid PC22(+)/P24

et al. 1989). Using *rbcS* from *S. tuberosum* as a probe, 85 out of 1000 plaques hybridized. One of these inserts is a 500-bp fragment with 75% amino acid homology to *rbcS* from potato (EMBL J03613, Wolter et al. 1988). These *Physcomitrella* sequences have been deposited in the EMBL data bank (accession numbers EMBL X76632, EMBL X76634).

### Southern analyses

Total DNA digested with *Eco*RI or *Hind*III, respectively, was hybridized to the homologous cDNA probes for *cab* or *rbcS*, respectively. The *cab* probe hybridized to 17 *Eco*RI fragments in the size range from 18.5 kb to 1 kb with one prominent signal, and to 14 *Hind*III fragments in the size range from 16.5 kb to 1.6 kb with four prominent signals (Fig. 5). The *rbcS* probe hybridized to 10 *Eco*RI-fragments varying in size from 12 kb to 3.2 kb with two prominent signals and to 9 *Hind*III fragments of 11 kb to 1.9 kb with two very prominent signals (Fig. 6). Although the intensities of the hybridization signals varied between the four genotypes, we could not

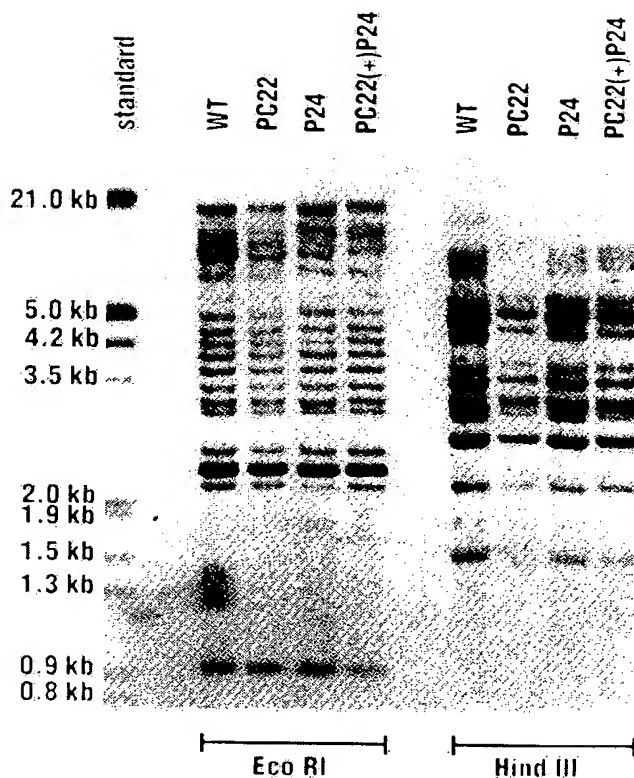


Fig. 5 Southern analyses of *Eco*RI- and *Hind*III-digested total DNA of four *Physcomitrella* genotypes hybridized to a *cab*-specific homologous probe. In 3 µg of total DNA the probe detects no restriction fragment length polymorphism between the wild type (WT), the two X-ray-derived mutants PC22 and P24 and their somatic hybrid PC22(+)/P24

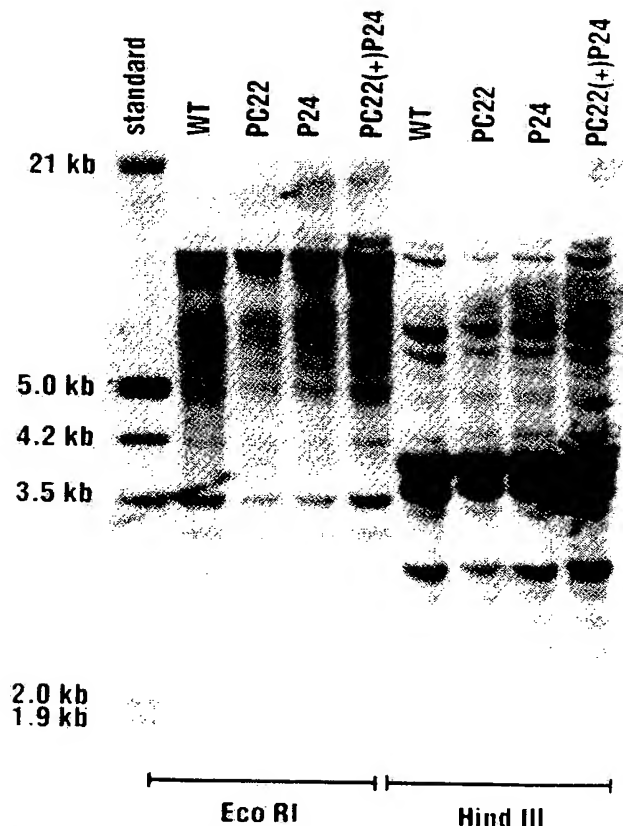


Fig. 6 Southern analyses of *Eco*RI- and *Hind*III-digested total DNA of four *Physcomitrella* genotypes hybridized to a *rbcS*-specific homologous probe. In 3 µg of total DNA the probe detects no restriction fragment length polymorphism between the wild type (WT), the two X-ray-derived mutants PC22 and P24 and their somatic hybrid PC22(+)/P24

find reproducible differences in the restriction patterns between them in repeated experiments.

## Discussion

Over several decades different chromosome numbers of *P. patens* have been reported: von Wettstein (1925) analysed a German isolate and suggested  $n=16$  (15–18), Bryan (1957) reported 27 mitotic chromosomes in an isolate from Michigan, while Engel (1968) illustrated by photographs of diakinesis  $n=14$  in an English isolate. Finally, Cove (1983) cited unpublished data from M. Newton revealing 26 chromosomes per haploid cell. Based on mitotic as well as on meiotic chromosome counts we support here Bryan's (1957) observation of  $n=27$  chromosomes. Discussing the discrepancies between von Wettstein's and her own results, Bryan (1957) suggested two possibilities: (1) the existence of two chromosomal races, or (2) clumped chromosomes. We frequently detected clumped chromosomes, resulting in fewer but larger chromosomal blocks. However, in the

photograph presented by Engel (1968) no such blocks can be seen, thus favouring the existence of two chromosomal races. Our strain is a descendant of the one propagated by Engel, but while our results do not agree with Engel's (1968) findings, they do coincide with those found by Bryan (1957). As her strain was collected in Michigan, the existence of two chromosomal races in *P. patens* seems an unlikely explanation for these obvious discrepancies in chromosome numbers.

Although there are numerous reports of mutant analysis via somatic hybridization in *Physcomitrella* and *Funaria* (Grimsley et al. 1977a, b; Watts et al. 1985; Mejia et al. 1988; Ye et al. 1989; Featherstone et al. 1990), no karyotypic analysis has been reported so far, from either mutants or fusants. A fusant, PC22(+)/P24, derived from one-to-one electrofusion of single *Physcomitrella patens* mutant protoplasts (Hansen et al. 1988), showed complementation to the wild type based on morphological, physiological and isoenzyme data (Ye et al. 1989). Furthermore, we presented indirect data for a massive fusion of nuclei within 1 h post-protoplast fusion (Rother et al. 1994). However, the karyological



data reported here provide the first direct evidence for somatic hybridization after fusion of moss protoplasts. The chromosomes were too numerous and too small to allow a more precise estimate of chromosome number than  $54 \pm 2$ . Thus, we cannot exclude the possibility that parts of one mutant genome might have been eliminated in this somatic hybrid, although chromosomal instability seems unlikely in intraspecific hybrids.

The small chromosomes observed were the first indication for a small genome size in *Physcomitrella*. In FCM analyses of DAPI-stained cells, we surprisingly found that every moss genotype examined has only one peak of fluorescence, although we analysed fast growing protonemata from agitated liquid culture. As moss protonemata grow by division of apical cells, we enhanced the number of dividing cells by subsequent subcultures, allowing us to analyse mitotic chromosomes. However, not more than one peak was ever discernible. Likewise, metaphase cells of the four moss genotypes were seldom found, despite the pretreatment, and could be accumulated best between 2.00 a.m. and 4.00 a.m. From this finding we conclude that we are working with rather synchronous cultures with cells probably being arrested in  $G_1$  or in  $G_2$  for most of the day. By comparison with *A. thaliana* (0.14 pg/2C) and *B. napus* (3.2 pg/2C), we calculated the DNA amounts in these peaks to be about 0.6 pg (WT, PC22), 1.2 pg (P24) and 1.6 pg (PC22(+)P24), respectively. If moss nuclei were arrested in  $G_1$  and thus (apart from the somatic hybrid), represented 1C, a *Physcomitrella* wild-type strain would have a haploid genome of about 600 Mbp, representing a genome size about eight times larger than *Arabidopsis*. However, Cove (1983) stated that, although "segregation ratios provide evidence of functional haploidy", chromosome numbers may suggest a polyploid state of *Physcomitrella*. Moreover, we used the A+T-specific fluorochrome DAPI, possibly overestimating DNA amounts of the A+T-rich moss nuclei. As we found 34.6% G+C for total DNA and 38.6% G+C for plastid DNA, it is reasonable that the G+C content of *Physcomitrella* nuclei is less than 34%, whilst *Arabidopsis* has 41.1% (Leutwiler et al. 1984) and the *Brassica* family between 41.2% and 45% G+C (Thomas and Sherratt 1956).

Although chromosome numbers did not differ between the wild type and the two mutants PC22 and P24, the latter had about double the amount of DNA per nucleus. One possible explanation is that P24 was arrested in  $G_2$ , another explanation being that the strain has accumulated repetitive DNA. To investigate the latter possibility, equal amounts of *RsaI*-digested total DNA were hybridized to the homologous probe for 25S rDNA and to sonicated wild-type DNA. These analyses provided evidence for at least one family of highly repetitive DNA in the moss. Furthermore, they revealed length homogeneity in the rDNA family, which is not common to all plant species (Ganal and Hemleben 1986). Additionally, the results indicated variable amounts of middle and highly repetitive DNA in the

four moss genotypes; this might be a result of the X-ray treatment in the case of the mutants, and a consequence of nuclei fusion in the somatic hybrid. However, these findings are unlikely to explain the 100% increase in nuclear DNA content of P24 relative to WT or to PC22. Obviously, detailed cell cycle analyses have to be undertaken, including G+C-specific fluorochromes to validate further the genome sizes of *Physcomitrella* and its mutants.

The chromosomes of *Physcomitrella* are too small to enable chromosomal rearrangements, which may occur after X-ray mutagenesis (Auerbach 1976) or somatic hybridization (Kumar and Cocking 1987; Templaar et al. 1991), to be visualized directly. Therefore, the four genotypes were subjected to RFLP analysis with homologous probes. Ideally, these probes should represent multigene families allowing analysis of several loci in one hybridization. Therefore, we constructed a cDNA library as a permanent source of homologous probes and isolated from that one *cab* and one *rbcS* cDNA clone by heterologous hybridization. About 5–7% of all clones were positive with each of these probes, indicating that these multigene families are as highly expressed in moss as they are in higher plants. Partial sequencing of these clones showed reasonable homologies to equivalent sequences from higher plants. Our RFLP data revealed that the *cab* and the *rbcS*-families comprise several genes, as in most higher plants. However, none of these experiments revealed differences in the hybridization patterns between the four genotypes, indicating that no massive chromosomal rearrangements have occurred, either after X-ray mutagenesis of spores or after somatic hybridization. Furthermore, our *cab* data were in accordance with those report of Long et al. (1989), showing a high degree of conservation at least in the genomic organization of this gene family between two cultivars of *P. patens*. From these experiments a further indication was obtained about the small size of its genome, as we needed less than 3 µg of total DNA for the detection of single-copy genes with non-radioactive hybridization.

The mosses *F. hygrometrica* and *P. patens* have been developed as model systems in plant physiology, especially due to the outstanding contributions of Bopp (1990) and Cove (1992). These led to the suggestion that a genetic dissection of *Physcomitrella* could make significant contributions to understanding the evolution of plant developmental processes (Goldberg 1988; Chasan 1992; Reski 1994). We have now shown that *Physcomitrella*, besides its well known advantages, has a fairly small genome and, furthermore, is amenable to the essential techniques of plant molecular biology. Taking these results into account, *P. patens* deserves more attention from plant molecular biologists interested in the evolution of regulatory processes.

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